Project 505.7000
Ontwikkeling en verbetering van onderzoekmethoden voor vlees en vleesprodukten (G. Cazemier)

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Vergelijking van een tijd besparende analysemethode voor hydroxyproline met ISO 3496-1978

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SAMENVATTING

De bepaling van hydroxyproline volgens de referentiemethode ISO 3496 is zeer tijdrovend. In een door het Nordisk Metodikkomitté för Livsmedel voorgestelde methode is een aantal wijzigingen ten opzichte van ISO 3496 aangebracht die de analysetijd per monster sterk verkorten. Om na te gaan of deze Scandinavische methode gebruikt zou kunnen worden als intern RIKILT voorschrift werd, na enige geringe aanpassingen van de methode, een aantal monsters met deze methode onderzocht en vergeleken met de met behulp van de ISO methode verkregen resultaten. De aanpassingen bestonden uit het gelijk maken van de monster- en reagentiavolumina en reactietijd aan die van de ISO methode. Het bleek dat in de meeste gevallen met de Scandinavische methoden lagere gehalten werden gevonden dan met de referentiemethode.
Het gemiddelde terugvindingspercentage (op basis van ISO=100%) van de Scandinavische methode is 93% met een variatiecoëfficient van 5,5%. Indien rekening wordt gehouden met dit terugvindingspercentage is het goed mogelijk deze methode als routinemethode te gebruiken.

1 INLEIDING

Voor het bepalen van de eiwitkwaliteit van vlees en vleeswaren is de hoeveelheid collageen een belangrijke parameter. Collageen is het hoofdbestanddeel van pezen en bindweefsel en wordt beschouwd als minderwaardig eiwit omdat het geen essentiële aminozuren bevat.
Het gehalte aan collageen wordt in de meeste gevallen colorimetrisch bepaald door het gehalte aan hydroxyproline (HYP), een van de bouwstenen van collageen, te meten. Bij het RIKILT worden hiervoor als referentiemethode ISO 3496-1978 (Bijlage A) en voor grotere series een auto-analysermethode gebruikt.
De ISO methode is gebaseerd op het volgende principe. HYP wordt uit het monster vrij gemaakt door ca. 2 gram monster gedurende 16 uur te hydrolyseren in 100 ml 6 N HCl dat 0,75% Sn Cl_2 bevat. De Sn Cl_2 dient om nevenreacties, zoals de vorming van eiwithoudende huminstoffen te beperken (Möhler 1969). Na filtratie wordt een gedeelte van het filtraat met loog met behulp van een pH meter op pH 8 gebracht, dit om het tin uit de oplossing te verwijderen. Deze op pH gebrachte oplossing wordt opnieuw gefiltreerd. Een gedeelte van het aldus verkregen filtraat wordt geoxideerd met behulp van gebufferde chlooramine-T en vervolgens gekleurd met dimethylaminobenzaldehyde. De kleurintensiteit wordt vergeleken met die van een ijkreeks.
Enkele bezwaren van de ISO methode zijn:

- Voor de hydrolyse zijn, bij grote series monsters veel verwarmingsapparaten nodig.
- Tijdens het hydrolyseren ontsnappen regelmatig zoutzuurdampen hetgeen aanslag en corrosie veroorzaakt.
- Het gebruik van een pH meter voor het op pH brengen kost veel tijd, evenals het filtreren van de op pH gebrachte oplossing.

Het Nordisk Metodikkommitté för Livsmedel (NMKL) stelde in 1987 een analysemethode voor die voornoemde nadelen niet heeft (Bijlage B). Deze methode komt overeen met een door Wyler (1972) gepubliceerde methode. Bij deze methode wordt het monster in een stoof bij 105°C gehydrolyseerd met zwavelzuur. Het hydrolyseren gebeurt zonder toevoeging van Sn Cl_2, waardoor het monster niet geneutraliseerd hoeft te worden. In grote lijnen is de verdere afwerking van de bepaling gelijk aan de

ISO-methode, alleen is de concentratie van de buffer anders, terwijl een kleiner deel van het hydrolysaat in bewerking wordt genomen. Uit een door het NMKL uitgevoerd ringonderzoek (Bijlage C), blijkt dat de door hen voorgestelde methode redelijk goede waarden geeft voor de herhaalbaarheid en de reproduceerbaarheid.
Volgens ISO 3496 mag het verschil tussen twee duplobepalingen niet groter zijn dan 5% van het rekenkundige gemiddelde. Uit bovenstaand en ander onderzoek (Jonas and Wood, 1983) blijkt dat deze eis niet bruikbaar is voor monsters met een HYP-gehalte <0,8%. Om na te gaan of de Scandinavische methode bruikbaaar is als intern RIKILT voorschrift wordt deze, in iets gewijzigde vorm, in dit verslag vergeleken met de ISO methode.
De door ons aangebrachte wijzigingen in de Scandinavische methode hadden betrekking op het colorimetrische gedeelte van de bepaling. De hoeveelheden monster en reagentia en de reactietijden werden gelijk gemaakt aan die van de ISO methode, dat wil zeggen er werden 2x de voorgeschreven hoeveelheden monster en reagentia gebruikt en de kleurontwikkelingstijd werd van 15 minuten op 20 minuten gebracht. Deze wijzigingen werden aangebracht om verwarring met de ISO methode, die in verband met wettelijke regelingen regelmatig door het RIKILT moet worden uitgevoerd, te voorkomen. Waar in het verslag sprake is van de Scandinavische methode, wordt de gewijzigde Scandinavische methode bedoeld, die beschreven is in RSV A0557.

2 MATERIAAL EN METHODEN

Vijftien monsters gehakt van verschillende herkomst werden gehomogeniseerd in een Moulinette. In deze monsters werd het HYP-gehalte in duplo bepaald volgens ISO 3496 en de Scandinavische methode. Verder werden 26 monsters kroketten, frikandellen en gehakt onderzocht met de beide methoden.

3 RESULTATEN EN DISCUSSIE

De analyseresultaten van de HYP-bepaling in 15 monsters gehakt met behulp van de twee methoden staan vermeld in tabel 1.
Voor beide methoden geldt, zoals ook reeds in het NMKL-verslag werd geconcludeerd, dat veel waarden niet aan de ISO-eis voldoen waarin

wordt gesteld dat het maximale verschil tussen duplo´s niet meer dan 5% relatief mag bedragen. De spreiding van de ISO methode bedroeg 0,022 g/100 g (Vr=5.4%), de herhaalbaarheid was 0,061 g/100 g.
Op één uitzondering na werden in alle gevallen met de ISO methode hogere gehalten gevonden dan met de Scandinavische methode, gemiddeld 0,028 g/ 100 g. De verhouding Scandinavisch/ISO is gemiddeld 0,929 $\pm$ 0,028.
Tabel 2 geeft de resultaten weer van de analyse van 26 monsters kroketten, frikandellen en gehakt die met de beide methoden zijn onderzocht. Van de volgens ISO bepaalde monsters zijn er acht in duplo uitgevoerd, de overige waarden zijn resultaten van simplo bepalingen. De met behulp van de Scandinavische methode gevonden waarden zijn allen de gemiddelden van duplobepalingen.
Met de ISO methode wordt nu gemiddeld 0,037 g/100 g meer gevonden, de verhouding Scandinavisch/ISO is vergelijkbaar met de in tabel 1 gevonden waarde, 0,907 $\pm$ 0,040. Deze iets slechtere waarden in vergelijking met het eerste onderzoek kunnen worden verklaard door het mogelijk minder homogeen zijn van de monsters en het in simplo uitvoeren van de meeste bepalingen volgens ISO.

4 CONCLUSIES

Vergeleken met de ISO methode levert bepaling van hydroxyproline met de Scandinavische methode een grote tijdswinst op.
Met de Scandinavische methode wordt gemiddeld 0,03 g/100 g hydroxyproline minder gevonden dan met de ISO methode.
Gemiddeld wordt met de Scandinavische methode 93%, met een variatie coëfficient van 5,5%, van de volgens ISO bepaalde waarde gevonden.
Indien rekening wordt gehouden met de gevonden verschillen tussen de beide methoden, is de Scandinavische methode goed bruikbaar als routinemethode.
De matige resultaten die met de Scandinavische methode werden gevonden ten opzichte van ISO 3496 geven, mede gezien de resultaten van later onderzoek (zie bijlage 5), aanleiding om de beide methoden met een groter aantal produkten van een grotere diversiteit te vergelijken.

Tabel 1: Bepaling van het gehalte aan hydroxyproline (g/100 g) van 15 monsters gehakt volgens ISO 3496 en de Scandinavische methode.

	ISO	Gemidd.	Scandinavisch	Gemidd.	ISO-Scand.	Scand./ISO
1	0,642-0,603	0,623	0,607-0,626	0,617	0,006	0,9904
2	0,458-0,471	0,465	0,407-0,425	0,416	0,049	0,8946
3	0,502-0,437	0,470	0,466-0,469	0,468	0,002	0,9957
4	0,368-0,415	0,392	0,397-0,368	0,383	0,009	0,9770
5	0,343-0,316	0,330	0,311-0,285	0,298	0,032	0,9030
6	0,455-0,481	0,468	0,423-0,473	0,448	0,020	0,9573
7	0,525-0,550	0,538	0,500-0,488	0,494	0,044	0,9182
8	0,239-0,257	0,248	0,256-0,226	0,241	0,007	0,9718
9	0,248-0,265	0,257	0,205-0,239	0,222	0,035	0,8638
10	0,348-0,310	0,329	0,341-0,322	0,332	-0,003	1,0091
11	0,211-0,194	0,203	0,171-0,208	0,190	0,013	0,9360
12	0,393-0,407	0,400	0,379-0,383	0,381	0,019	0,9525
13	0,500-0,533	0,517	0,425-0,433	0,429	0,088	0,8298
14	0,332-0,345	0,338	0,313-0,262	0,288	0,050	0,8521
15	0,475-0,465	0,470	0,410-0,423	0,417	0,053	0,8872
				Gemiddeld	0,0283	0,92924
				Stand dev.	0,02502	0,056000

Tabel 2: Bepaling van het hydroxyproline gehalte (g/100 g) in 26 monsters vleesprodukten volgens ISO 3496 en de Scandinavische methode.

Soort monster	ISO	Scandinavisch*	ISO-Scand.	Scand./ISO
Kroket	0,207	0,166	0,041	0,8019
Kroket	0,032	0,032	0	1,0000
Frikandel	0,204	0,182	0,022	0,8922
Frikandel	0,214	0,228	-0,014	1,0654
Frikandel	0,322	0,344	-0,022	1,0683
Frikandel	0,270	0,218	0,052	0,8074
Frikandel	0,348	0,313	0,035	0,8994
Frikandel	0,288*	0,273	0,015	0,9479
Frikandel	0,258*	0,255	0,003	0,9884
Frikandel	0,366*	0,344	0,022	0,9399
Frikandel	0,533*	0,490	0,043	0,9193
Gehakt	0,330	0,334	-0,004	1,0121
Gehakt	0,488	0,416	0,072	0,8525
Gehakt	0,456	0,424	0,032	0,9298
Gehakt	0,448	0,362	0,086	0,8080
Gehakt	0,431	0,440	-0,009	1,0209
Gehakt	0,295	0,204	0,091	0,6915
Gehakt	0,290	0,286	0,004	0,9862
Gehakt	0,442	0,381	0,061	0,8620
Gehakt	0,315	0,290	0,025	0,9206
Gehakt	0,443	0,442	0,001	0,9977
Gehakt	0,446	0,385	0,061	0,8632
Gehakt	0,489*	0,440	0,049	0,8998
Gehakt	0,360*	0,330	0,030	0,9167
Gehakt	0,554*	0,432	0,122	0,7798
Gehakt	0,480*	0,344	0,136	0,7167
		Gemiddeld	0,0367	0,9072
		Stand dev.	0,0405	0,0991

* Gemiddelde resultaten van duplo bepalingen

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BIJLAGE A

INTERNATIONAL STANDARD ISO 3496

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION•МЕЖДУНАРОДНАЯ ОРГАНИЗАЦИЯ ПО СТАНДАРТИЗАЦИИ•ORGANISATION INTERNATIONALE DE NORMALISATION

Meat and meat products — Determination of L(−)-hydroxyproline content (Reference method)

Viande et produits à base de viande — Détermination de la teneur en L(−)-hydroxyproline (Méthode de référence)

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Price based on 3 pages

Meat and meat products — Determination of L(−)-hydroxyproline content (Reference method)

1 SCOPE AND FIELD OF APPLICATION

This International Standard specifies a reference method for the determination of the L(−)-hydroxyproline content of meat and meat products.

2 REFERENCE

ISO 3100, *Meat and meat products — Sampling.*

3 DEFINITION

L(−)-hydroxyproline content of meat and meat products : The amount of L(−)-hydroxyproline determined according to the procedure specified in this International Standard, and expressed as a percentage by mass.

4 PRINCIPLE

Hydrolysis of a test portion in constant-boiling hydrochloric acid solution containing tin(II) chloride. Filtration and dilution of the hydrolysate. Neutralization, with sodium hydroxide, of an aliquot portion of the diluted hydrolysate. Filtration and dilution. Oxidation of the L(−)-hydroxyproline by chloramine-T, followed by the formation of a red compound with *p*-dimethylaminobenzaldehyde. Photometric measurement at a wavelength of 558 nm.

5 REAGENTS

All reagents shall be of recognized analytical quality. The water used shall be distilled water or water of at least equivalent purity.

5.1 Hydrochloric acid solution containing tin(II) chloride.

Dissolve 7,5 g of tin(II) chloride dihydrate ($SnCl_2.2H_2O$) in water, dilute to 500 ml and add 500 ml of hydrochloric acid (ρ_{20} 1,19 g/ml).

5.2 Hydrochloric acid, approximately 6 M solution.

Mix equal volumes of hydrochloric acid (ρ_{20} 1,19 g/ml) and water.

5.3 Sodium hydroxide, approximately 10 M solution.

Dissolve 40 g of sodium hydroxide in water. Cool and dilute to 100 ml.

5.4 Sodium hydroxide, approximately 1 M solution.

Dissolve 4 g of sodium hydroxide in water. Cool and dilute to 100 ml.

5.5 Buffer solution, pH 6,0.

Dissolve in water :

50 g of citric acid monohydrate ($C_6H_8O_7.H_2O$);

26,3 g of sodium hydroxide;

146,1 g of sodium acetate trihydrate [$Na(CH_3CO_2).3H_2O$].

Dilute to 1 000 ml with water. Mix this solution with 200 ml of water and 300 ml of propan-1-ol.

This solution is stable for several weeks at 4 °C.

5.6 Chloramine-T reagent

Dissolve 1,41 g of *N*-chloro-*p*-toluenesulphonamide, sodium salt trihydrate (chloramine-T) in 10 ml of water and successively add 10 ml of propan-1-ol and 80 ml of the buffer solution (5.5).

Prepare this solution immediately before use.

5.7 Colour reagent

Dissolve 10,0 g of *p*-dimethylaminobenzaldehyde in 35 ml of perchloric acid solution [60 % (*m/m*)] and then slowly add 65 ml of propan-2-ol.

Prepare this solution on the day of use.

NOTE — If purification of the *p*-dimethylaminobenzaldehyde is necessary (see note to 8.5), proceed as follows :

Prepare a saturated solution of the *p*-dimethylaminobenzaldehyde in hot 70 % (*V/V*) ethanol. Cool, first at room temperature and finally in a refrigerator. After about 12 h, filter on a Buchner funnel. Wash with a little 70 % (*V/V*) ethanol. Again dissolve in hot 70 % (*V/V*) ethanol. Add cold water and agitate thoroughly. Repeat this procedure until a sufficient quantity of milk-white crystals has been formed. Place in the refrigerator overnight. Filter on the Buchner funnel, wash with 50 % (*V/V*) ethanol and vacuum dry over phosphorus(V) oxide.

5.8 **L(−)-Hydroxyproline standard solutions**

Prepare a stock solution by dissolving 50 mg of 4-hydroxypyrrolidine-α-carbonic acid (hydroxyproline) in water. Add 1 drop of the hydrochloric acid solution (5.2) and dilute to 100 ml with water. This solution is stable for at least one month at 4 °C.

On the day of use, dilute 5 ml of the stock solution to 500 ml with water in a volumetric flask. Then prepare four standard solutions by diluting 10 – 20 – 30 and 40 ml of this solution to 100 ml with water to obtain L(−)-hydroxyproline concentrations of 0,5 – 1 – 1,5 and 2 µg/ml respectively.

6 APPARATUS

Ordinary laboratory apparatus, and in particular :

6.1 Mechanical meat mincer, laboratory size, fitted with a plate with holes not exceeding 4 mm in diameter.

6.2 Round or flat bottom hydrolysis flask, capacity about 200 ml, wide-necked, equipped with an air-cooled or water-cooled condenser.

6.3 Electric heating device (for example, heating mantle, hot-plate, or electrically heated sand bath).

6.4 Filter paper disks, diameter 12,5 cm[1].

6.5 pH meter.

6.6 Aluminium or plastic foil.

6.7 Water bath, capable of being thermostatically controlled at 60 ± 0,5 °C.

6.8 Spectrophotometer, capable of being used at a wavelength of 558 ± 2 nm, or **photoelectric colorimeter** with an interference filter with absorption maximum at 558 ± 2 nm.

6.9 Glass cells of 10 mm optical path length.

6.10 Analytical balance.

7 SAMPLE

7.1 Proceed from a representative sample of at least 200 g. See ISO 3100.

7.2 Store the sample in such a way that deterioration and change in composition are prevented.

8 PROCEDURE

8.1 Preparation of test sample

8.1.1 *Raw meat and raw meat products*

Reduce intact meat to small cubes (approximately 0,5 cm^3, i.e. length of side approximately 8 mm) by cutting it while it is cold (just below 0 °C), using a sharp knife.

Either place the sample in a container and seal the latter hermetically, or vacuum pack the sample in heat-resistant plastic film; then heat so as to maintain a temperature of at least 70 °C for at least 30 min; cool and proceed as in 8.1.2.

During the remaining stages of preparation of the test sample and the weighing out of the test portions, ensure that the sample is kept well mixed and, in particular, that any fat or fluid is kept evenly distributed.

NOTE – The heat treatment softens the raw connective tissue and makes it less resistant to homogenization by mincing. However, it may also lead to separation of a fluid containing gelatine. The presence of fat may also demand special attention for the production of a homogeneous test sample.

8.1.2 *Cooked meat and cooked meat products*

Make the sample homogeneous by passing it at least twice through the meat mincer (6.1), and mixing. Keep the homogenized sample in a completely filled, air-tight, closed container and store it in such a way that deterioration and change in composition are prevented. Analyse the test sample as soon as possible, but always within 24 h.

8.2 Test portion

Weigh, to the nearest 0,001 g, about 4 g of the test sample into the hydrolysis flask (6.2). Ensure that none of the sample adheres to the side wall of the flask.

8.3 Hydrolysis

8.3.1 Place some silicon carbide boiling chips in the flask and add 100 ± 1 ml of the hydrochloric acid solution containing tin(II) chloride (5.1). Heat to gentle boiling using the heating device (6.3), and maintain for 16 h under reflux (conveniently overnight).

NOTE – If desired by the analyst, the hydrolysis may alternatively be accomplished in two periods, each of 7 to 8 h, on consecutive days. This alternative procedure has been proved experimentally to yield results that are not significantly different from those obtained with a single hydrolysis period of 16 h.

8.3.2 Filter the hot hydrolysate through filter paper (6.4) into a 200 ml one-mark volumetric flask. Wash the flask and filter paper three times with 10 ml portions of hot hydrochloric acid solution (5.2) and add the washings to the hydrolysate. Make up to the mark with water and mix.

1) For example, S and S No. 287 and Whatman GF/A are suitable.

Continue the determination as soon as possible, but at the latest on the day after hydrolysis.

8.4 Colour development and measurement of absorbance

8.4.1 Using a pipette, transfer into a beaker a volume V ml of the hydrolysate (8.3.2) so that, after dilution to 250 ml (see 8.4.2), the L(−)-hydroxyproline concentration will be within the range 0,5 to 2 µg/ml.

NOTE – In most cases, V will be of the order of 5 to 25 ml depending on the amount of connective tissue present in the sample.

8.4.2 With the aid of the pH meter (6.5), adjust the pH to 8 ± 0,2 by the addition, drop by drop, first of the 10 M sodium hydroxide solution (5.3) and then, when approaching the required pH, of the 1 M sodium hydroxide solution (5.4). Filter the contents of the beaker into a 250 ml one-mark volumetric flask. Wash the beaker and the tin hydroxide precipitate on the filter paper at least three times with 30 ml portions of water, collecting the washings in the volumetric flask. Make up to the mark with water and mix.

8.4.3 Transfer 4,00 ml of this solution into a test tube and add 2,00 ml of the chloramine-T reagent (5.6). Mix and leave at room temperature for 20 ± 1 min.

8.4.4 Add 2,00 ml of the colour reagent (5.7), mix thoroughly and cap the tube with aluminium or plastic foil (6.6).

8.4.5 Transfer the tube quickly into the water bath (6.7), controlled at 60 ± 0,5 °C, and heat for exactly 20 min.

8.4.6 Cool under running tap water for at least 3 min.

8.4.7 Measure the absorbance at 558 ± 2 nm in a glass cell (6.9) against water, using the spectrophotometer or the photoelectric colorimeter equipped with an interference filter (6.8).

8.4.8 Subtract the absorbance measured for the blank solution (8.5) and read the L(−)-hydroxyproline concentration of the diluted hydrolysate from the calibration graph obtained as described in 8.6.

8.5 Blank test

Carry out in duplicate the procedure described in 8.4.3 to 8.4.8 inclusive, substituting water for the diluted hydrolysate.

NOTE – If the absorbance of the blank exceeds 0,040, a fresh colour reagent (5.7) should be prepared and, if necessary, the *p*-dimethylaminobenzaldehyde should be purified (see note to 5.7).

8.6 Calibration graph

8.6.1 Carry out the procedure described in 8.4.3 to 8.4.8 inclusive, substituting in turn 4,00 ml of each of the four diluted standard L(−)-hydroxyproline solutions (5.8) for the diluted hydrolysate.

8.6.2 Plot the measured absorbance values, corrected for the blank value, against the concentrations of the standard L(−)-hydroxyproline solutions, and construct the best-fitting straight line through the plotted points and the origin.

NOTE – It is necessary to prepare a new calibration graph for each series of analyses.

8.7 Duplicate values

Carry out the procedure on duplicate test portions.

9 EXPRESSION OF RESULTS

9.1 Method of calculation and formula

For each of the two test portions, calculate the L(−)-hydroxyproline content, as a percentage by mass, from the formula

$$\frac{5\,c}{m \times V}$$

where

c is the L(−)-hydroxyproline concentration, in micrograms per millilitre, of the diluted hydrolysate (8.4.2) as read from the calibration graph;

m is the mass, in grams, of the test portion (8.2);

V is the volume, in millilitres, of the aliquot portion of the hydrolysate taken for dilution to 250 ml (see 8.4.1).

Take as the result the arithmetic mean of the two calculated values, provided that the requirement of 9.2 is satisfied. Report the result to the nearest 0,01 %.

9.2 Agreement between duplicates

The difference between the two calculated values obtained simultaneously or in rapid succession from the duplicate test portions by the same analyst shall not exceed 5 % of the arithmetic mean value.

10 TEST REPORT

The test report shall show the method used and the result obtained. It shall also mention any operating conditions not specified in this International Standard, or regarded as optional, as well as any circumstances that may have influenced the result.

The report shall include all details necessary for complete identification of the sample.

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12.3.8 HYDROXYPROLINE - COLORIMETRIC DETERMINATION AS A MEASURE OF COLLAGEN IN MEAT AND MEAT PRODUCTS

1. Scope and field of application

A quantitative method for the determination of hydroxyproline and estimation of collagenous connective tissue in meat and meat products.

2. Definition

Collagenous connective tissue contains 12.5% (alternatively 14.0%) hydroxyproline. Other muscle proteins contain only small amounts or no hydroxyproline.

3. Principle

Hydrolysis of the sample in sulphuric acid at 105°C. Filtration and dilution. Oxidation of hydroxyproline with chloramine T. Development of a red-purple colour by addition of 4-dimethylaminobenzaldehyde and photometric measurement at a wavelength of 560 nm.

4. Reagents

All reagents should be of analytical quality. The water used should be distilled or of equivalent purity.

4.1 Sulphuric acid, approx. 7N

Add 750 ml of water in a 2000 ml volumetric flask. Add slowly, agitating continuously, 375 ml of concentrated sulphuric acid (1.84 g/ml). Cool to room temperature and dilute to the mark with water.

4.2 Buffer solution, pH 6.0

Dissolve 30 g of citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$), 15 g of sodium hydroxide and 90 g of sodium acetate trihydrate ($CH_3COONa \cdot 3 H_2O$) in about 500 ml of water. Transfer solution quantitatively to a 1000 ml volumetric flask. Add 290 ml of propan-1-ol. Check the pH and adjust, if necessary, with acid or alkali solution. Dilute to the mark with water.

This solution is stable for about 2 months at +4°C in a dark bottle.

4.3 Oxidant solution

Dissolve 1.41 g of chloramine-T reagent in 100 ml of water.

This solution is stable for one week at +4°C in a dark bottle.

4.4 Colour reagent

Dissolve 10 g of 4-dimenthylaminobenzaldehyde in 35 ml of perchloric acid [60% (m/m)]. Add slowly, agitating continously, 65 ml of propan-2-ol.

This solution should be prepared on the day of use.

4.5.1 Hydroxyproline - stock solution 600 µg/ml

Dissolve 60 mg hydroxyproline in water in a 100 ml volumetric flask. Dilute to the mark with water.

This stock solution is stable for about 2 months at +4°C.

4.5.2 Hydroxyproline - intermediate solution 6 µg/ml

Pipette 5 ml of stock solution (4.5.1) in a 500 ml volumetric flask. Dilute to the mark with water.

This solution should be prepared on the day of use.

4.5.3 Hydroxyproline - standard solutions

Pipette 10, 20, 30 and 40 ml of intermediate solution (4.5.2) in 100 ml volumetric flasks. Dilute to the mark with water. These standard solutions will contain 0.6 - 1.2 - 1.8 and 2.4 µg hydroxyproline per ml.

These solutions should be prepared on the day of use.

5. Apparatus

5.1 pH meter

5.2 Mechanical meat chopper with plate openings of 2 and 3 mm.

5.3 Food processor, approx. 1500-3000 r/min.

5.4 Erlenmeyer, wide-necked, 100 ml

5.5 Watch glass, diameter 5-6 cm

5.6 Drying oven, 105 ± 1°C

5.7 One-mark volumetric flasks of 100 ml, 500 ml, 1 l and 2 l

5.8 Funnels

5.9 Filter paper discs, Munktell OB or OOR, diameter 12.5 cm

5.10 One-mark pipettes of 1, 2, 5, 10, 20 and 40 ml

5.11 Test tubes of 10 ml. Use the same kind of test tubes for each measuring chain.

5.12 Water bath, with temperature control, 60 ± 0.5°C

5.13 Spectrophotometer or photo-electric colorimeter with an interference filter.
558 ± 2 nm. Glass cells of 10 mm optical path length.

6. Sampling and sample preparation

6.1 Sampling

Use a representative sample of at least 200 g.

Put the sample into a plastic bag, an airtight container or similar airtight wrapping. Store the sample at max. +5°C until further treatment or at max. -18°C if the sample is to be stored for longer than three days.

6.2 Sample preparation

Remove the sample quantitatively from the bag. Gravy, jelly, fat or anything else which has separated from the product in the package must be included.

Thaw frozen material in a refrigerator. Chop the sample immediately after removing from the refrigerator.

Cut the sample into small pieces. Pass the sample twice through a chopper (5.2) with plate openings of 2 or 3 mm. In the case of very fatty samples, use 3 mm plate openings to prevent the sample from greasing in the chopper. Mix thoroughly after each grinding. Alternatively, the sample can be homogenized in a food processor (5.3). Samples with a very loose consistency can be mashed and mixed well with a fork. The homogeneity of the minced sample can be controlled by adding a spoonful of charcoal powder to the chopper or food processor before starting homogenizing and afterwards checking the colour of the material.

Store the sample in an airtight container at max. +5°C, or at max. -18°C if the sample is to be stored for longer than three days.

7. Procedure

7.1 Hydrolysis

Weigh, to the nearest 0.001 g, about 4 g of the sample into the erlenmeyer (5.4). Avoid that the sample adheres to the side wall of the flask. Perform duplicates. Add 30 ml of the sulphuric acid (4.1). Cover the erlenmeyers with watch glasses (5.5) and place them in the drying oven (5.6) at 105 ± 1°C for 16 h (conveniently overnight).

Transfer the hot hydrolysate quantitatively into a 500 ml volymetric flask. Wash the erlenmeyer with water. Dilute to the mark with water and mix. Filter a part of the solution through filter paper (5.9) into a 100 ml erlenmeyer.

This filtrate is stable for at least two weeks at 4°C. Dilute the filtrate with water so that the hydroxiproline concentration of this final dilution will be within the range 0.5 to 2.4 µg/ml. In most cases, the dilution of 5 ml of filtrate to 100 ml will be suitable.

7.2 Colour development

Transfer 2.00 ml of the hydrolysate solutions to test tubes (5.11) by means of a pipette. Transfer to two test tubes 2.00 ml of water instead of the hydrolysate solutions (Blank test). Add to each test tube 1.00 ml of the oxidant solution (4.3). Mix thoroughly and leave the samples at room temperature for 20 ± 2 min.

Add to each test tube 1.00 ml of the colour reagent (4.4) and mix thoroughly. Cap the tubes with aluminium or plastic foil or screw cap. Place the tubes immediately in the water bath (5.12) at 60 ± 0.5°C for exactly 15 min. Cool the tubes under running tap water for at least 3 min.

7.3 Measurement

Dry the tubes. Measure the absorbance of the solutions in 10 mm glass cells using a spectrophotometer or a photoelectric colorimeter (5.12) at 558 ± 2 nm against a blank.

7.4 Calibration curves

Prepare a calibration curve for each series of measurement. Transfer 2.00 ml of each standard solution (4.5.3) to a test tube and continue as described above (7.2 and 7.3). Draw a standard curve with the absorbance as y and the amount of hydroxyproline (1.2 - 2.4 - 3.6 and 4.8 µg) as x.

8. Expression of results

8.1 Calculation of the hydroxyproline content

Calculate the hydroxyproline content of the sample (H), expressed as g per 100 g, using the formula:

$$H = \frac{h \times 2.5}{m \times V}$$

h = the amount of hydroxyproline in µg per 2 ml hydrolysate solution, read from the calibration curve;

m = the mass of sample used in g;

V = ml filtrate taken for dilution to 100 ml (7.1).

Take as the result the arithmetic mean of the two calculated values for each sample, providing that the requirement for repeatability (8.3) is satisfied. Express the results to the nearest 0.01%.

8.2 Collagenous connective tissue content

Calculate the collagenous connective tissue content of the sample (B), expressed as g per 100 g, using the formula:

$$B = H \times 8$$

REMARK - Collagenous connective tissue contains 12.5% HPRO, if its nitrogen content is multiplied by 6.25.

8.2.1 Collagenous connective tissue content per crude protein

Calculate the collagenous connective tissue content per crude protein (BR), expressed as g per 100 g, using the formula:

$$BR = \frac{B \times 100}{\%\ \text{crude protein (i.e. N x 6.25)}}$$

N = the nitrogen content of the sample, expressed as g per 100 g.

8.2.2 Remarks

Several countries use a different calculation, which is based on that collagenous connective tissue contains 14% hydroxyproline if its nitrogen content is multiplied by the factor 5.55. This is the correct nitrogen factor for collagen. The corresponding hydroxyproline factor will in this case be 7.1 instead of 8.

8.3 Reliability

The reliability of the method is stated as an equation for the relation between the hydroxyproline content and the repeatability resp. reproducibility of the sample.

8.3.1 Repeatability (r)

$r = 0.0131 + 0.0322 \times H$

8.3.2 Reproducibility (R)

$R = 0,0195 + 0,0529 \times H$

H = the hydroxyproline content of the sample

8.3.3 Application of repeatability

The difference between two calculated values obtained simultaneously or in rapid succession from the duplicate test portions by the same analyst shall not exceed the calculated difference according to 8.3.1.

Arithmetical example:

Suppose that the hydroxyproline content has been analysed and the arithmetic mean value of a double determination is 0.50 g hydroxyproline per 100 g sample.

The repeatability is calculated according to 8.3.1.

$$r = 0.0131 + 0.0322 \times 0.50$$

$$r = 0.029$$

The difference between the two determinations shall in this example not exceed $\pm$0.029% from the mean value 0.50.

9. Literature

ISO. International Organization for Standardization. ISO 3496 - 1978. Meat and meat products - Determination of L(-)-hydroxiproline content (Reference method).

§ 35 LMBG. Amtliche Sammlung von Untersuchungsverfahren. Untersuchung von Lebensmitteln. Bestimmung des Hydroxiprolingehaltes in Fleisch und Fleischzeugnissen. L-06.00-8, 1980. Beuth Verlag GmbH, Berlin, Köln.

WYLER, O. Routine-Untersuchungsmethoden für Fleisch und Fleischwaren. 2. Mitteilung: Die Bestimmung des kollagenen Bindegewebes durch vereinfachte Ermittlung des Hydroxiprolingehaltes. Die Fleischw. 52(1), 1972: 42-44.

KÖTTFORSKNINGSINSTITUTET
KÄVLINGE

Swedish Meat Research Institute

BIJLAGE C

Vårt datum/Our date Vår ref./Our ref.

18 January 1988
KK/NMK 1373F

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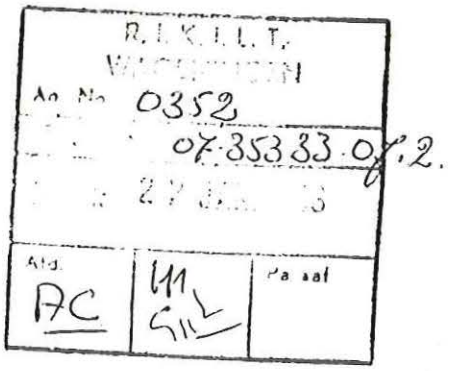

Dear Dr. van der Veen,

Subject: Collaborative results for the determination of hydroxyproline

First I apologize for not having informed you earlier of the collaborative results. I planned to send you a copy of an article in the AOAC-journal but this article will not be finished before spring 1988, depending on several unexpected reasons.

I hope you will accept a summary of the Swedish report until the AOAC-article is accessible.

Sample preparation

Eighteen laboratories took part in the collaborative study. Each laboratory received eight samples:

1. Frozen falu sausage
2. Frozen sirloin
3. Sample No. 2 + 0.8 g HPRO per 100 g sample
4. Frozen blood sausage
5. Identical with sample No. 1
6. Freeze-dried ham
7. Freeze-dried rind
8. A mixture of samples 6 and 7 in the ratio 5:2

All samples were homogenized in a Robot-Coupe food processor. The freeze-dried material was furthermore homogenized after drying in a mill. The samples were distributed in a frigolite box containing four freeze-elements (-70°C).

We recommended the following sample amounts to be analysed: 4 g frozen material and 1.5 g freeze-dried material (addition of 2.5 g of water). The collaborators were asked to analyse the samples as duplicates but to report the values of the single determinations.

Postadress	Postal address	Gatuadress	Telefon	Telex	Telefax	Telegram	Postgiro	Bankgiro
Box 504 244 00 KÄVLINGE	POB 504 S-244 00 KÄVLINGE SWEDEN	V. Långgatan 20 KÄVLINGE	046/73 22 30	32206 (Meatres S)	046-736137	Meatresearch LUND	567 10-7	283-9322

Results

Collaborator 14 observed big particles indicating incomplete hydrolysis. Therefore these values have not been included in the report.

The results of the collaborative study are shown in Tables 1 and 2.

The results were calculated according to guidelines of Pocklington (prepared for IUPAC Commission VI.3 and ISO/TC 34/SC 11, July 1986. LGC, London).

'Outlying' results were calculated according to Cochrans test (repeatability) and Dixons test (reproducibility) at at 5% significance level.

All values could be accepted according to Cochrans test. Sample 3 and 6 from collaborator 14 and sample 4 from collaborator 8 were Dixon-outliers. These results were omitted from further calculations. Mean values and standard deviations respectively coefficients of variation for repeatability (r) and reproducibility (R) have been calculated for each sample (Tables 1 and 2).

Samples 1 and 5 are indentical. The mean values agree excellently (0.245 respectively 0.251 g HPRO/100 g). Student's t-test showed no significant difference between the samples (5% sign. level).

The average recovery for sample 3 was 96.1% - an acceptable level.

The hydroxyproline content of the 'known' sample 8 was calculated to 1.42 g HPRO/100 g and does agree well with the analysed result 1.40 g/100 g.

Repeatability and reproducibility

In comparison with other collaborative studies for determination of hydroxyproline in meat, based on the ISO-method (1978), the results of the present study are quite acceptable (Lord & Swan, 1986; Jonas & Wood, 1983). The present study shows an even lower repeatability and reproducibility for freeze-dried material in comparison with Jonas & Wood's results.

According to ISO (1978), the difference between two calculated values obtained simultaneously or in rapid succession from the duplicate test portions by the same analyst shall not exceed 5% of the arithmetric mean value.

The above mentioned studies and the present study are showing that the ISO-recommendation is not usable if the sample contains less than approx. 0.8% HPRO.

Repeated analysis in our laboratory showed that the freeze-dried material was less homogenous than the frozen material. This concerns above all sample 8 - a mixture with different particle size.

The present method concerns mostly analysis of fresh material - not freeze-dried. The present study includes enough fresh material to permit elimination of the results from the freeze-dried material with the aim to receive lower repeatability and reproducibility. The following equations are based on results from only the fresh material.

Repeatability depending on hydroxyproline content:

$r = 0.0131 + 0.0322 \times$ HPRO-content

Reproducibility depending on hydroxyproline content:

$R = 0.0195 + 0.0529 \times$ HPRO-content

Sweden, Denmark and Finland have accepted the present method as an official NMKL method. The Norwegian National Committee has not answered yet.

I hope you will be satisfied with this preliminary report.

Your laboratory has No. 14 in the Tables.

Thank you for your collaboration.

Thanking you in advance,

Yours sincerely,

SWEDISH MEAT RESEARCH INSTITUTE
Analytical Services

Kurt Kolar

Encls.

Literature

ISO. International Organization for Standardization. ISO 3496 - 1978. Meat and meat products - Determination of L(-)-hydroxyproline content (Reference method).

JONAS, D.A. & WOOD, R. Determination of hydroxyproline in meat products: Collaborative Study.
J. Assoc. Publ. Analysts 21, 1983: 113-121.

LORD, D.W. & SWAN, K.J. Determination of hydroxyproline in Meat Products: Collaborative trial.
J. Assoc. Publ. Analysts 24, 1986:69-72.

POCKLINGTON, W.D. Guidelines for the development of standard methods by collaborative study. Prepared for IUPAC Commission VI.3 and ISO/TC 34/SC 11. 2nd edition, July 1986. LGC, London. ISBN 0 948926 00 7.

Table 1. Collaborative results (g/100 g) for determination of hydroxyproline.

Coll.	Samples							
	1 Falu sausage		2 Sirloin		3 Sirloin		4 Blood sausage	
1	0,26	0,25	0,11	0,11	0,86	0,88	0,40	0,40
2	0,25	0,25	0,11	0,11	0,89	0,89	0,40	0,39
3	0,25	0,25	0,11	0,12	0,88	0,86	0,41	0,41
4	0,23	0,23	0,11	0,10	0,90	0,89	0,38	0,36
5	0,24	0,24	0,10	0,09	0,85	0,83	0,39	0,38
6	0,25	0,24	0,11	0,12	0,92	0,90	0,38	0,38
7	0,24	0,24	0,10	0,10	0,86	0,87	0,39	0,38
8	0,23	0,25	0,10	0,10	0,91	0,90	0,43[b]	0,47[b]
9	0,25	0,25	0,11	0,11	0,89	0,86	0,40	0,39
10	0,25	0,26	0,11	0,10	0,90	0,91	0,39	0,38
11	0,26	0,25	0,10	0,11	0,86	0,83	0,39	0,40
12	0,25	0,24	0,10	0,10	0,87	0,86	0,36	0,37
13	0,26	0,26	0,13	0,11	0,85	0,87	0,39	0,41
14	0,26	0,23	-[a]	-[a]	0,98[b]	0,99[b]	0,42	0,40
15	0,24	0,23	0,08	0,09	0,87	0,85	0,36	0,39
16	0,24	0,24	0,11	0,10	0,87	0,91	0,37	0,38
17	0,24	0,24	0,11	0,11	0,87	0,89	0,40	0,39
18	0,23	0,25	0,11	0,11	0,86	0,84	0,39	0,39
Mean	0,245		0,106		0,875		0,389	
Added, g/100 g					0,800			
Av. rec., g/100 g					0,769			
Av. rec., %					96,1			
Repeatability, std dev.	0,008		0,006		0,014		0,009	
Repeatability CV, %	3,3		5,6		1,7		2,4	
Reproducibility std dev.	0,010		0,009		0,023		0,015	
Reproducibility CV, %	4,0		8,8		2,7		3,8	
Repeatability (r)	0,023		0,017		0,041		0,027	
Reproducibility (R)	0,028		0,026		0,066		0,041	

[a] Incomplete hydrolysis (some particles left).
[b] Rejected by Dixon's test. Values not used in calculation.

Table 2. Collaborative results (g/100 g) for determination of hydroxyproline.

Coll.	Samples							
	5 Falu sausage		6 Ham		7 Rind		8 Ham + rind	
1	0,26	0,26	0,43	0,43	4,07	4,08	1,46	1,49
2	0,25	0,26	0,42	0,43	4,04	4,10	1,42	1,48
3	0,27	0,26	0,44	0,42	4,16	4,19	1,40	1,48
4	0,24	0,24	0,37	0,39	4,12	4,08	1,40	1,37
5	0,24	0,23	0,40	0,40	3,87	3,82	1,38	1,50
6	0,22	0,22	0,39	0,36	3,79	3,75	1,42	1,30
7	0,25	0,25	0,40	0,40	3,84	3,90	1,37	1,36
8	0,26	0,25	0,43	0,42	4,02	3,96	1,45	1,33
9	0,25	0,25	0,39	0,40	4,19	4,11	1,44	1,42
10	0,24	0,25	0,36	0,38	4,07	3,97	1,39	1,35
11	0,28	0,27	0,38	0,37	3,58	3,63	1,30	1,29
12	0,25	0,25	0,38	0,37	3,71	3,86	1,31	1,34
13	0,26	0,26	0,44	0,43	4,16	4,22	1,54	1,56
14	0,24	0,26	0,23[a]	0,24[a]	4,44	4,34	1,38	1,49
15	0,25	0,24	0,35	0,35	3,75	3,80	1,25	1,27
16	0,24	0,25	0,37	0,38	4,03	4,02	1,34	1,31
17	0,26	0,26	0,43	0,41	4,03	4,12	1,44	1,41
18	0,25	0,26	0,45	0,45	4,08	4,04	1,40	1,45
Mean	0,251		0,391		4,00		1,40	
Repeatability, std dev.	0,006		0,010		0,049		0,046	
Repeatability CV, %	2,4		2,5		1,2		3,3	
Reproducibility std dev.	0,013		0,049		0,194		0,077	
Reproducibility CV, %	5,1		12,4		4,9		5,5	
Repeatability (r)	0,027		0,027		0,137		0,129	
Reproducibility (R)	0,036		0,138		0,549		0,218	

[a] Rejected by Dixon's test. Values not used in calculation.

OPMERKINGEN OVER DE HERHAALBAARHEID

Door omstandigheden lag er een grote tijd tussen het schrijven en het verschijnen van dit rapport. In de tussentijd werd een groot aantal monsters met de voorgestelde methode onderzocht. Hierbij bleek dat de herhaalbaarheid van de methode kleiner was dan de in het verslag genoemde 0,055 g/100 g. Mogelijke oorzaak voor dit verschil zou het verschil in onderzochte produkten kunnen zijn. De in het verslag genoemde r was ontleend aan onderzoek van 15 monsters gehakt, de later onderzochte monsters waren 36 monsters frikandel, 25 monsters pluisvlees uit kroketten en 10 monsters diversen (verschillende soorten worst en vleeswaren). Voor deze produkten werden de volgende waarden gevonden.

		Gehalte HYP (%)				
Soort	Aantal	Laagste	Hoogste	Gemidd.	s g/100g	r g/100g
Frikandel	36	0,167	0,866	0,312	0,0156	0,044
Kroket	25	0,039	0,489	0,130	0,0082	0,023
Diversen	10	0,127	0,425	0,240	0,0100	0,028
Totaal	71	0,031	0,866	0,206	0,0127	0,036

Bovenstaand geeft aanleiding om de herhaalbaarheid van de methode te veranderen van 0,06 tot 0,04 g/100 g. Verder lijkt het zinvol om de vergelijking met ISO 3496 te herhalen met een aantal verschillende monsters.